

Evaluation of the Effects of Chlorophyllin on Apoptosis Induction, Inhibition of Cellular Proliferation and mRNA Expression of *CASP8*, *CASP9*, *APC* and β -catenin

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Abstract: Chlorophyllin is a semi-synthetic derivative of chlorophyll with antioxidant and antimutagen properties that controls the enzymes involved in the metabolism of xenobiotics and in the induction of apoptosis. In this study, we evaluated the effects of chlorophyllin on apoptosis induction, inhibition of cell proliferation, and gene expression in the human colorectal adenocarcinoma cell line HT29. Chlorophyllin significantly reduced cell survival after 48 h at 100 $\mu\text{g/mL}$ and after 24 h at 500 or 1,000 $\mu\text{g/mL}$, respectively based on both MTT cytotoxicity and cell proliferation kinetics assays. These effects were dose dependent. Chlorophyllin did not induce apoptosis after 24 h at any concentration. Chlorophyllin downregulated the cell cycle genes *APC* and β -catenin (*CTNBI*) but did not affect the expression of apoptotic induction genes in the extrinsic pathway (*CASP8*) or the intrinsic pathway (*CASP9*). At the studied concentrations, the inhibitory effect of chlorophyllin on cell growth was directly related to the regulation of β -catenin gene expression and not to *APC* expression, because *APC* was mutated and inactive. The studied concentrations suggest no potential for chlorophyllin as an apoptosis inducer based on cytomorphological changes or gene expression changes of the studied caspases.

Key words: *APC* gene, β -catenin, chlorophyllin, *CTNNB* gene, HT29 cells, proliferation

INTRODUCTION

Recently, there has been a growing interest in the identification of food components and their derivatives that can prevent cancer. One of these components is chlorophyllin, which has a long history of therapeutic use in traditional medicine. Chlorophyllin is a semi-synthetic derivative of chlorophyll in which the central magnesium atom is replaced with copper and the phytol esters are replaced with sodium, making it soluble in water (Sarkar *et al.*, 1994). As a result of these changes, chlorophyllin is more stable than chlorophyll (Sarkar *et al.*, 1995) because it is more resistant to light and heat. Similarly, it is used worldwide as a food dye.

Chlorophyllin has been widely studied due to its beneficial biological effects, including wound healing (Edwards, 1954) and anti-inflammatory properties (Larato and Pfau, 1970), treatment of kidney stones by controlling calcium oxalate crystals (Tawashi *et al.*, 1980) and as an internal deodorant (Young and Beregi, 1980). Although these applications illustrate the potential for chlorophyllin use in clinical settings, its role in cancer chemoprevention has gained most of the attention. This pigment has shown significant *in vitro* and *in vivo* biological effects consistent with cancer prevention, such as antimutagenic

effects, antioxidant effects, and regulating detoxification and apoptosis induction (Ferruzzi and Blakeslee, 2007).

Its antimutagenic effects and the absence of significant toxicity in humans and animals (Ong *et al.*, 1986) make chlorophyllin an ideal chemopreventive compound. *In vitro*, this molecule can protect against the mutagenic effects of direct and indirect dietary and environmental mutagens, such as heterocyclic amines (Dashwood *et al.*, 1991), benzo(a) pyrene (Arimoto *et al.*, 1993; Ferruzzi *et al.*, 2002), aflatoxin (Dashwood *et al.*, 1991), heavy metals (Olvera *et al.*, 1993) and ionizing radiation (Abraham *et al.*, 1994; Madrigal-Bujaidar *et al.*, 1997; Santosh Kumar *et al.*, 1999). Antimutagenic effects of chlorophyllin and other vegetable extracts have been demonstrated in several model systems: *Salmonella typhimurium* (Bronzetti *et al.*, 1990), *Drosophila* (Negishi *et al.*, 1989), and mammalian cell cultures (Bez *et al.*, 2001; Rampazo *et al.*, 2002; Negraes *et al.*, 2004).

Chlorophyllin inhibits carcinogenesis in trout, rats, and mice by forming complexes with several mutagenic agents in the diet (Dashwood, 1997). Egner *et al.* (2001) reported that chlorophyllin consumption in Chinese populations reduced the level of aflatoxin-DNA adducts in urine, which may lead to a reduced risk of developing liver cancer in humans.

In addition to being an effective carcinogenesis inhibitor, chlorophyllin is capable of inducing apoptosis and inhibiting cell proliferation. In HCT116 human colon cancer cells, chlorophyllin induces apoptosis by activating caspase-8 (Diaz *et al.*, 2003). Likewise, chlorophyllin induces apoptosis and inhibits cell proliferation in MCF-7 cells (Chiu *et al.*, 2005); however, the mechanisms have not been completely elucidated.

Recently, the chemoprotective ability and the mechanisms of action of chlorophyllin in apoptosis and cell proliferation have been investigated to develop new pharmacological agents for cancer prevention. For this reason, this study evaluated the roles of chlorophyllin in apoptosis induction and inhibition of cell proliferation as well as the possible mechanisms involved in these cellular processes by analyzing the expression of genes related to apoptosis (*CASP8* and *CASP9*) and cell proliferation (*APC* and β -catenin (*CTNBI*)).

MATERIALS AND METHODS

Chemical agents: Chlorophyllin (Sigma-Aldrich) was dissolved in D-MEM (Gibco) culture medium and filter-sterilized (Millex[®] 0.22 μ m, Millipore). The positive controls agents were doxorubicin (CAS 25316-40-9, Adriblastina[®], Pharmacy) in the MTT cytotoxicity assay (10 μ g/mL) cisplatin (CAS 15663-27-1, Sigma-Aldrich) in the cell proliferation kinetics and cell viability assays (1 μ g/mL) and camptothecin (CAS 7689-03-4, Acros Organics) in the apoptosis induction assay (10 μ g/mL).

Cell culture: The colorectal adenocarcinoma cell line (HT29) used in this study was obtained from the Rio de Janeiro Cell Bank. These cells were cultured in 25 cm² culture flasks in D-MEM supplemented with 10% fetal bovine serum (Gibco) and an antibiotic/antimycotic (0.1%) at 37°C and 5% CO₂. This study was conducted in Laboratory Toxicological Genetics 2011 in the State University of Londrina, Londrina, Paraná, Brazil.

Cytotoxicity assay: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed following the protocol first described by Mossmann (1983). The assays were performed in a 24-well cell culture plate with 3×10^4 cells seeded in each well, except for the control wells without cells. Cells were cultured for 24 h to allow attachment. The culture medium was then discarded, and fresh medium with the following treatments was added for 24, 48, or 72 h, respectively: control (culture media), doxorubicin at a concentration of 10 μ g/mL, and chlorophyllin at 100, 500, or 1,000 μ g/mL, respectively. After treatment, the culture medium was discarded, and fresh media containing 0.167 mg/mL MTT was added to each well. The plate was incubated for 4 h. Following incubation, the MTT-containing medium was

removed from the wells and dimethyl sulfoxide was added to dilute the crystals that had formed. The samples were read in a microplate reader at 550 nm. The experiments were performed in triplicate. The following equation was used to calculate the percentage of cell survival:

$$\text{survival (\%)} = \frac{\text{TS} - \text{BS}}{\text{CS} - \text{BS}}$$

Legend:

TS = Mean absorbance of the treated samples
BS = Mean absorbance of the blank samples
CS = Mean absorbance of the control samples

Cell proliferation kinetics assay: For the proliferation kinetic analysis, 1.3×10^5 cells were seeded into each culture tube (10 cm²) with one of the following treatments: control (culture media), anti-proliferative agent (cisplatin 1 μ g/mL), and chlorophyllin at either 100 or 500 μ g/mL. After each treatment period (24, 48, or 72 h, respectively), the culture media and the two 2.5 Ml Phosphate-Buffered Saline (PBS) washes were saved. Then, cells were trypsinized for approximately 5 min, inactivated with the saved media, and centrifuged for 5 min at 1,080 rpm. The supernatant was discarded, leaving only 1 mL. Cell counts after collection by trypsinization were performed in a Neubauer chamber. A proliferation kinetics curve was generated at the determined time points. The experiment was performed in three repetitions.

Cell viability assay-trypan blue: For the cell viability analysis, 1.3×10^5 cells were seeded into each experimental flask. Manual counting in a Neubauer chamber and Trypan blue dye exclusion techniques were used. This assay was performed concomitantly with the cell proliferation kinetics assay. After obtaining 1 mL of cell suspension in the cell proliferation kinetics assay, 20 μ L of this suspension was mixed with an equal volume of Trypan blue. Therefore, the dilution factor was 2. Next, 20 μ L of Trypan blue-containing suspension was placed on the chamber surface. Then, cells were counted under an optical microscope. The assay was performed in three repetitions.

Assay for evaluating apoptosis induction: A six-well plate containing a coverslip (18 \times 18 mm) in each well was seeded with 1.6×10^5 cells. After 24 h of culture to allow the cells to adhere to the coverslip, the following treatments were tested: control (culture media), apoptosis inducer (camptothecin 10 μ g/mL) and two concentrations of chlorophyllin (100 and 500 μ g/mL). After treatment, cells were collected following the procedure described by Rovozzo and Burke (1973). Briefly, cells were washed with PBS, and the coverslips were removed from the culture plate and fixed in Carnoy fixative for 5 min. The

Table 1: Sequences of the primers used for real-time PCR

Gene	Primers	References/accession # (NCBI)
GAPDH	F:5'ACAAGATTGTGAAGG TCG GTG TCA 3' R:5'AGCTTCCCATTCTCAGCCTTGACT 3'	(Sugaya <i>et al.</i> , 2005)
CASP8	F:5'GCAAAAGCACGGGAGAAAGT 3' R:5'TGCATCCAAGTGTGTTCCATT3'	(Castaneda and Rosin-Steiner, 2006)
CASP9	F:5'GCTCTTCCTTTGTTTCATCTCC 3' R:5'GTTTCTAGGGTTGGCTTCG 3'	(Chen <i>et al.</i> , 2009)
APC	F:5'AAAGCGCCATGATATTGCACGGTC 3' R:5'TGTTTGCTGTGCTCACGTTTCCAG 3'	Constructed/ M 74088
CTNNB1	F:5'CCTATGCAGGGGTGGTCAAC3' R:5'CGACCTGGAAAACGCCATCA3'	Constructed/NP-064633

coverslips were dipped in plates containing decreasing concentrations of ethanol (95 to 25%), followed by a wash in McIlvaine buffer for 5 min, staining with acridine orange (0.01%, 5 min), and another wash in buffer. The coverslips were inverted onto a slide containing a drop of buffer and sealed with nail polish. The slide was analyzed under a fluorescent microscope (420-490 nm excitation filter and a 520 nm barrier filter). The experiments were performed in three repetitions, and 1,500 cells were analyzed per treatment. Apoptotic cells were identified by analyzing morphological changes in cells, including chromatin condensation, formation of apoptotic bodies, or nuclear DNA fragmentation after acridine orange staining.

Quantitative real-time reverse-transcription PCR (qRT-PCR): A qRT-PCR experiment was performed following instructions provided in the guidelines of the Minimum Information for Publication of Quantitative RT-PCR Experiments MIQE (Bustin *et al.*, 2009). First, 10⁶ cells were seeded in 25 cm² culture flasks, and cultures were established for 24 h. After the incubation period, the treatments (control, chlorophyllin) were performed in duplicate. Total RNA was isolated using Trizol-LS reagent (Invitrogen) after 12 h of treatment following the manufacturer's instructions. The samples chosen for the experiment were those with a 260 nm/280 nm absorbance ratio between 1.9 and 2.1. Sufficient quantities of intact genomic RNA were run on a 0.8% agarose gel and were used to synthesize cDNA. Two microliters of DNTPs (10 mM; Invitrogen), 1 µL oligo(dT) (10 pmol/µL; Invitrogen), and 500 ng genomic RNA were mixed and brought to a volume of 14.9 µL with DEPC water (1%). The reaction was incubated for 5 min at 65°C in a thermocycler (TECHNE TC 412) and rapidly transferred to ice. Four microliters of Mlv 5x buffer (Invitrogen), 0.1 µL ribonuclease inhibitor (RNase out, Invitrogen), and 1 µL reverse transcriptase (M-Mlv-RT, Invitrogen) were then added. The total volume was 20 µL. Finally, the reaction was incubated in a thermocycler at 37°C for 50 min and at 70°C for 15 min. The obtained cDNA was stored in a 80°C freezer. Real-time PCR was performed in a PTC 200 DNA Engine Cycler with a Chromo 4 detection system (MJ Research-BIO RAD). Amplification was detected by measuring fluorescence emission from

SYBR Green (Platinum SYBR Green qPCR Supermix-UDG, Invitrogen) upon binding to double-stranded DNA. The thermocycler conditions were as follows: cDNA denaturation at 50°C for 1 min and 95°C for 3 min, followed by 35 cycles at 95°C for 20 sec, primer annealing at 60°C for 30 sec, and elongation at 72°C for 20 sec, followed by 95°C for 10 sec and 40°C for 1 min. A melting curve analysis was performed at the end of the reaction with 0.5°C steps from 50 to 95°C for 5 sec each. Data were normalized with primers for glyceraldehyde-3-phosphodehydrogenase (*GAPDH*) amplified in conjunction with the experiment. The primers used in the PCR experiments are shown in Table 1. All experiments were performed on two independent cultures, and each cDNA sample was analyzed in triplicate for each primer.

Statistical analysis: Data from the MTT cytotoxicity assay were analyzed using analysis of variance (ANOVA) followed by Tukey's test ($\alpha = 0.05$) using the GraphPad Prism5 program. Data obtained in the cell proliferation kinetics, cell viability and apoptosis induction assays were analyzed using ANOVA followed by Dunnett's test ($\alpha = 0.05$) using GraphPad Prism5. The expression levels were determined by the method of Pfaffl *et al.* (2002) and analyzed with REST-384 software.

RESULTS

Analysis of cytotoxicity: As demonstrated by the MTT assay, chlorophyllin decreased the cell survival rate in a dose-dependent manner (Fig. 1). At 24 h, only the 100 µg/mL concentration had a survival rate equivalent to the control or, in other words, was not cytotoxic. All concentrations (100, 500, and 1000 µg/mL, respectively) significantly decreased the survival rate compared to controls at 48 and 72 h. Therefore, they were cytotoxic. Based on these results, the 100 and 500 µg/mL concentrations were chosen for the other assays.

To better understand the cytotoxic effects of the chosen chlorophyllin concentrations (100 and 500 µg/mL), we evaluated the cell proliferation kinetics at 24, 48 and 72 h, respectively. Figure 2 shows that 100 µg/mL chlorophyllin did not significantly change the HT29 cell proliferation rate at 24 h. However, 500 µg/mL

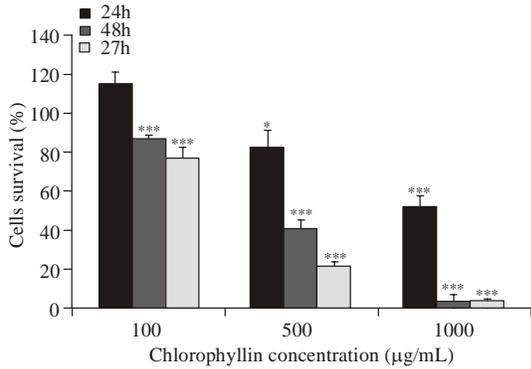


Fig. 1: Cell survival percentages. Calculations are based on absorbance levels obtained from the MTT test on HT29 cells treated with chlorophyllin for 24, 48, or 72 h (control = 100%); Error bars represent the mean±standard deviation from three independent experiments; *: p<0.05; ***: p<0.001 compared to control

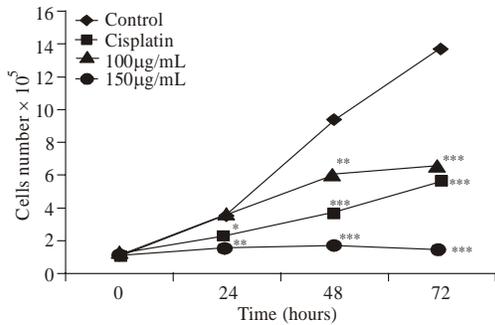


Fig. 2: Cell proliferation kinetics. Total cell counts were obtained by counting HT29 cells in a Neubauer chamber after 24, 48 and 72 h of chlorophyllin treatment. Cisplatin: 1 µg/mL; *: p<0.05; **: p<0.01; ***: p<0.001 compared to control

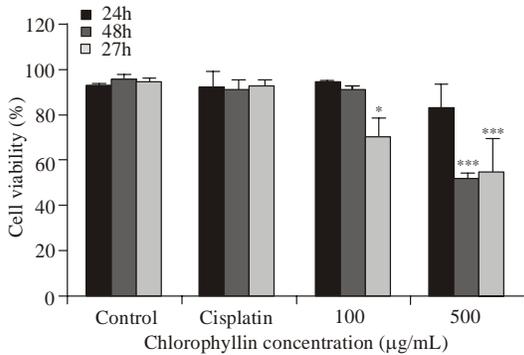


Fig. 3: Percentage of cell viability in HT29 cells treated with chlorophyllin for 24, 48 or 72 h. Cisplatin: 1 µg/mL; Error bars represent mean±standard deviation of three independent experiments; *: p<0.05; ***: p<0.001 compared to control

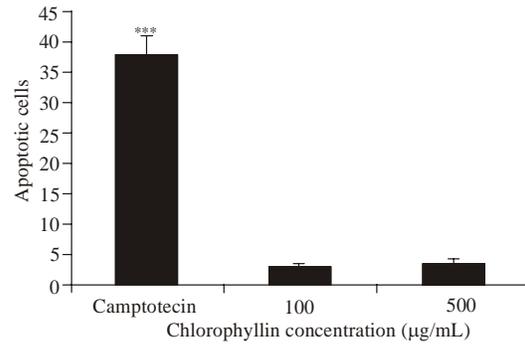


Fig. 4: Average number of apoptotic cells identified by acridine orange staining in HT29 cells treated with chlorophyllin for 24 h. Camptotecin: 10 µg/mL; ***: p<0.001 compared to control

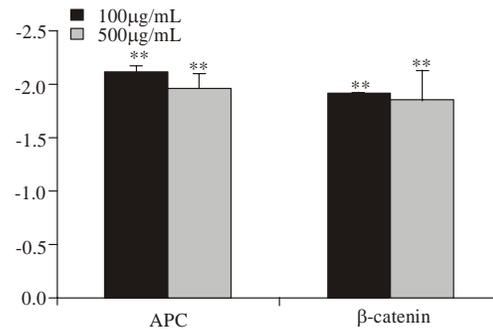


Fig. 5: Analysis of the relative expression of the APC and β-catenin genes by RT-PCR in HT29 cells after 12 h of chlorophyllin treatment at 100 or 500 µg/mL; **: p<0.01 compared to control (the statistical analysis was done using REST-384 software)

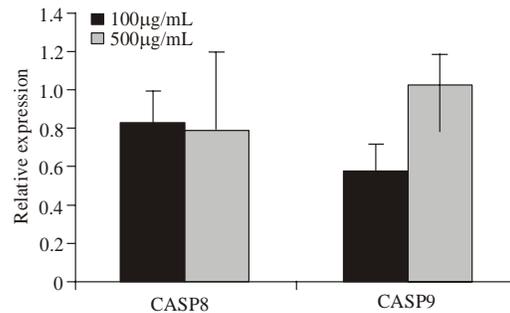


Fig. 6: Analysis of CASP8 and CASP9 expression in HT29 cells by RT-PCR after 24 h of chlorophyllin treatment at 100 or 500 µg/mL; **: p<0.01 compared to control (the statistical analysis was done using REST-384 software)

chlorophyllin significantly decreased cell proliferation at all time points. This reduction was dose-dependent at the studied concentrations. The positive control (cisplatin 1 µg/mL) was significantly cytotoxic compared to the negative control after 24 h of treatment.

To determine if the antiproliferative effects observed in the proliferation kinetics assay were due to cell death or to decreased cell proliferation, we analyzed cell viability. The results in Fig. 3 show that a concentration of 100 µg/mL at 24 and 48 h and 500 µg/mL at 24 h induced a survival rate greater than 80% and were similar to the control. However, the higher concentration promoted statistically lower cell viability rates at 48 and 72 h, as did the 100 µg/mL concentration at 72 h.

Apoptosis induction analysis: Figure 4 shows the results from the apoptosis induction analysis after 24 h of chlorophyllin treatment at concentrations of 100 and 500 µg/mL. No concentrations induced apoptosis, as the results were similar to the negative control. No apoptotic cells were seen in the negative control. Conversely, the positive control (camptothecin 10 µg/mL) significantly induced apoptosis compared to the negative control after 24 h of treatment.

Gene expression: To further investigate the antiproliferative effects of chlorophyllin, we analyzed the expression of genes involved in cell proliferation, namely *APC* and β -catenin (*CTNNB1*), and genes involved in apoptosis, namely *CASP8* and *CASP9*, in HT29 cells after 12 h of chlorophyllin treatment (100 or 500 µg/mL) by qRT-PCR.

Analysis of relative expression levels, normalized to the constitutive gene *GAPDH*, showed that chlorophyllin treatment significantly decreased the expression of the *APC* gene compared to control (Fig. 5). This decrease was 2.1 times lower with a concentration of 100 µg/mL and 1.99 times at 500 µg/mL. The expression of *CTNNB1* was also significantly lower, with a 1.98-fold decrease at 100 µg/mL and 1.86-fold lower at 500 µg/mL (Fig. 5).

Figure 6 shows the relative expression of the *CASP8* and *CASP9* genes. Chlorophyllin treatments (100 and 500 µg/mL) did not significantly affect gene expression compared to the control.

DISCUSSION

Chlorophyllin is a semi-synthetic derivative of chlorophyll belonging to the porphyrin class of compounds, and it is commonly used as a food dye. It is also part of a group of phytochemicals implicated in cancer prevention (Ferruzzi and Blakeslee, 2007). The chlorophyll of green vegetables and its derivative, chlorophyllin, have antigenotoxic effects against several mutagens *in vitro*, as well as anticarcinogenic effects *in vivo* (Dashwood, 1997) and in clinical studies (Egner *et al.*, 2001).

To determine the appropriate concentrations to be used, we performed an MTT cytotoxicity assay. This is a test used to determine the cytotoxicity of substances, and it is one of the mostly used and most sensitive methods for cytotoxicity detection *in vitro* (Fotakis and Timbrell,

2006). However, this assay, which is commonly referred to simply as a cytotoxicity assay, not only evaluates cell death but can also show cell growth inhibition or cytostatic effects.

In this study, we showed that a minimum chlorophyllin concentration of 500 µg/mL was necessary for cytotoxicity at 24 h. This concentration reduced the cell survival rate by approximately 20%. Cytotoxicity was observed starting at 48 h for the lower concentration. Currently, the Food and Drug Administration allows three 200 mg chlorophyllin tablets to be ingested daily. This dose has been effective in preliminary studies of populations at risk for aflatoxin B1 exposure (Egner *et al.*, 2001), but little is known about the actual concentrations *in situ*, systemically, or in the gastrointestinal tract of this oral dose. Rats do not exhibit toxic effects from long-term exposure to chlorophyllin concentrations as high as 1-3% in the diet, which produce plasma chlorophyllin concentrations of 56 to 116 µg/mL (Harrison *et al.*, 1954).

Data from the cell proliferation kinetics study, which showed the cell growth curve after different treatment periods, were consistent with the results from the MTT assay. In this analysis, the highest concentration diminished cell proliferation starting at 24 h of exposure. At the lowest concentration, cell growth was inhibited at 48 h. Therefore, chlorophyllin had an antiproliferative effect on tumor cells.

Likewise, the proliferation assay showed that chlorophyllin induced a dose-dependent decrease in cell growth, but viability analysis demonstrated that cell processes were responsible for this inhibition. The cell viability assay, performed concurrently with the cell proliferation kinetics assay, showed that more than 80% of cells were viable in samples with the highest chlorophyllin concentration at 24 h and in samples with the lowest concentration at 48 h. This pattern indicates that the inhibition was not due to apoptosis or changes in cell permeability but to apoptosis-independent inhibition of cell proliferation. Lower concentrations of chlorophyllin have cytostatic effects, while higher concentrations are cytotoxic in mouse myeloma cell lines (Chernomorsky *et al.*, 1997). This outcome may indicate that cytostatic effects of chlorophyllin used as a co-adjuvant with a chemotherapeutic agent that causes cell death may have beneficial effects on cancer treatment. In acute treatments, chlorophyllin would be used to reduce cell growth with low toxicity and could be used for longer times, while chemotherapy would promote cancer cell death.

Given the inhibitory effects of chlorophyllin, we investigated the molecular mechanism of this compound by studying gene expression levels of *APC* and *CTNNB1*. *APC* regulates cell division by binding to β -catenin and targeting it for degradation in the proteasome (Wang *et al.*, 2006). β -Catenin, which is present in the cytoplasm, binds to and activates transcription factors that promote cell division (e.g., cyclin-D1). β -Catenin

translocation to the nucleus normally occurs in response to external signals that promote proliferation (Yochum *et al.*, 2007). However, mutation of the *APC* gene causes a loss of β -catenin control, leading to increased cytoplasmic concentrations and translocation to the nucleus. *APC* is mutated in the majority of colon cancers, including HT29 cells, resulting in a non-functioning protein, which, in turn, leads to uncontrolled cell proliferation (Morin *et al.*, 1996).

Chlorophyllin decreased the expression of both *APC* and *CTNNB1* at both tested concentrations. Based on these results, we propose that chlorophyllin decreases cell proliferation mainly by decreasing the expression of the β -catenin gene (a promoter of proliferation). This outcome leads to a decrease in *APC* gene expression. *APC* inhibition is downstream of decreased β -catenin expression due to low demand for this protein and a decreased need for its regulation. According to Carter *et al.* (2004), proteomic studies may improve our understanding of this mechanism.

In human HL-60 (promyelocytic leukemia), K-562 (myelogenous leukemia), MCF-7 (breast carcinoma), and mouse S-180 (sarcoma) cells, a dose-dependent decrease in cell density or cell growth has been observed after 72 h of chlorophyllin treatment at 25, 50, 100, 200 or 400 $\mu\text{g/mL}$, respectively (Chiu *et al.*, 2003). Similarly, the authors showed a decrease in cyclin-D1 and cyclin-E by western blot and flow cytometry, respectively, in MCF-7 cells treated with chlorophyllin at either 200 or 400 $\mu\text{g/mL}$ for 72 h, suggesting a possible mechanism for cell cycle control (Chiu *et al.*, 2003). The decrease in cyclin-D1 expression might be downstream of decreased β -catenin expression, as seen in our study.

Reduced cell proliferation and viability may not only be due to cytotoxic or cytostatic effects on cells or their genetic material but could also be due to induction of apoptosis. Diaz *et al.* (2003) reported that chlorophyllin leads to apoptosis in the colon cancer cell line HTC116 at concentrations of 62.5, 125, 250 and 500 μM (45-360 $\mu\text{g/mL}$), as identified by morphological observations and increased protein levels of caspase-8, but not caspase-9. This pattern suggests that the extrinsic apoptotic pathway is activated in this type of cancer.

Chiu *et al.* (2005) have studied the potential of chlorophyllin as an apoptotic inducer at 200 and 400 $\mu\text{g/mL}$ in MCF-7 breast cancer cells. Their results show that only the highest concentration (400 $\mu\text{g/mL}$) caused increased apoptosis at 24, 48 or 72 h, respectively. In addition, their study noted an antiproliferative effect at the highest concentration of chlorophyllin by depleting cyclin-D1, a cell cycle protein.

We analyzed two chlorophyllin concentrations (100 and 500 $\mu\text{g/mL}$) through morphological analysis under fluorescent microscopy and through gene expression analysis of caspase-8 and-9. The experimental results from our analysis of morphological changes in cells, such as chromatin condensation, formation of apoptotic bodies,

and increased caspase expression, show that none of the studied concentrations induces apoptosis. These differences between our results and those from previous studies could be due to the different cell lines used in each study, showing that different tissues and organs do not respond in the same way to chlorophyllin treatment and that some types of cancer are resistant to apoptotic cell death.

Based on our results, we propose that the cytotoxicity detected in the MTT assay is not due to cell death; instead, it might be attributed to decreased cell proliferation (cytostatic effect), which was confirmed by our assays. To conclude, chlorophyllin has an inhibitory effect on cell proliferation in HT29 cells at the studied concentrations, possibly due to decreased β -catenin expression. Moreover, chlorophyllin does not induce apoptosis in this cell line at 24 h, and it has a cytostatic, not cytotoxic, effect.

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